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Title: From Mitochondria To Healthy Aging: The Role Of Branched-chain Amino Acids Treatment: MATeR A Randomized Study

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Abstract

Rationale: Malnutrition often affects elderly patients and significantly contributes to the reduction in healthy life expectancy, causing high morbidity and mortality. In particular, protein malnutrition is one of the determinants of frailty and sarcopenia in elderly people.

Methods: To investigate the role of amino acid supplementation in senior patients we performed an open-label randomized trial and administered a peculiar branched-chain amino acid enriched mixture (BCAAem) or provided diet advice in 155 elderly malnourished patients. They were followed for 2 months, assessing cognitive performance by Mini Mental State Examination (MMSE), muscle mass measured by anthropometry, strength measure by hand grip and performance measured by the Timed Up and Go (TUG) test, the 30 seconds Chair Sit to Stand (30-s CST) test and the 4 meters gait speed test. Moreover we measured oxidative stress in plasma and mitochondrial production of ATP and electron flux in peripheral blood mononuclear cells.

Results: Both groups improved in nutritional status, general health and muscle mass, strength and performance; treatment with BCAAem supplementation was more effective than simple diet advice in increasing MMSE (1.2 increase versus 0.2, p=0.0171), ATP production (0.43 increase versus -0.1, p=0.0001), electron flux (0.50 increase versus 0.01, p<0.0001) and in maintaining low oxidative stress. The amelioration of clinical parameters as MMSE, balance, four meter walking test were associated to increased mitochondrial function.

Conclusions: Overall, our findings show that sustaining nutritional support might be clinically relevant in increasing physical performance in elderly malnourished patients and that the use of specific BCAAem might ameliorate also cognitive performance thanks to an amelioration of mitochondria bioenergetics.

Keywords

Malnutrition, elderly patients, branched-chain amino acids, muscle mass and strength, mitochondrial activity and biogenesis, oxidative stress.

Abbreviations

30-s CST, 30 seconds chair sit to stand test; ADL, activity of daily living; BCAAem, branched chain amino acid enriched mixture; BCAAs, branched chain amino acids; BMI, body mass index; CIRS, cumulative index rating scale; COX-1 and 4, cytochrome C oxidase 1 and 4; FOXO, forkhead box O; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDS, geriatric depression scale; GLM, linear regression models; MFN-1 and 2, mitofusin-1 and 2; MMSE, mini-mental state examination; MNA, mini nutritional assessment test; mTOR, mechanistic target of rapamycin; NRF-1, nuclear respiratory factor-1; NO, nitric oxide; OECD, organisation for economic co-operation and development; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; RT-PCR, real time PCR; TBARs, thiobarbituric acid reactive substances; TFAM, mitochondrial transcription factor A; TUG, timed up and go test.

Introduction

Thanks to an increased life expectancy, an improvement in health status and medical services, the older population is constantly increasing. In 2015, 617 million (8.5%) people in the world were aged 65 and over (older adults) and these numbers are estimated to rise to 1.6 billion by 2050 (1). Despite the increase in life expectancy, there is no corresponding increase in healthy life expectancy: recent findings in 2015 show that, despite a life expectancy at the age of 65 of 21.2 years for women and 17.9 years for men, only 9.4 years are healthy years (2). Concomitantly, health maintenance in older age will be one of the most relevant societal challenges in the future years. Lifestyle changes appear to be fundamental in increasing healthy life expectancy, and adequate nutrition is enormously important, given that malnutrition (i.e., undernutrition), particularly as protein-energy deficit is very common amongst the elderly population. This is due to the effects of aging *per se* that causes decreased salivation, difficulty swallowing, and delayed emptying of the stomach and oesophagus, as well as slower gastrointestinal movement (3). Other conditions associated with aging, such as drug use, loneliness, depression, lack of oral health, low quality of life, in addition to chronic non-communicable diseases, markedly increase the undernutrition risk (4). It has been estimated that undernutrition affects between 20 and 50% of hospitalised patients (5) and 5 and 10% of patients living at home in the community (6), the great variations reported in different studies depends not only to differences in the population analysed, but also on the adopted definition. Recently a large study (7), that applies harmonized criteria to define malnutrition in different clinical settings and population, shows a great underestimation of the problem and confirms higher prevalence of malnutrition in residents of nursing homes and hospitalized patients evaluated by Mini Nutritional Assessment test (MNA). Malnutrition is more prevalent in patients affected by acute and chronic disorders with reduced functional status (7) and is associated with poor clinical outcomes and prognosis. Malnutrition is associated with reduced immune function, anaemia, impaired cognitive function, and higher hospitalisation rate and is a strong independent predictor of mortality [for a review, see A. Granic et al, 2018 (8)]. Malnutrition causes body weight loss and muscle atrophy, decreased muscle strength and function, impaired balance, and increased fall and fall-related injuries. The malnutrition-linked muscle atrophy accelerates transition to frailty (9) and has been considered as one of the determinants of sarcopenia (10,11). Importantly, sarcopenia is defined as low muscle mass, with defective muscle strength (also named dynapenia) and decline of physical performance (12) and is, *per se*, associated with increased morbidity and mortality (13). Although several guidelines and consensus documents on nutritional care of malnourished elderly subjects have been proposed (14), and protein needs established in the range from 0.8 g/kg/day (healthy adults) and up to 1.5 g/kg/day (in some cases even higher) according to age, disease and degree of protein depletion (15), the daily protein consumption in older subjects is often inadequate and undernutrition and sarcopenia are underestimated and considered as one of the factors of aging (4, 7).

It has been suggested that the aging process significantly affects protein metabolism and enhances the muscle wastage that accompanies undernutrition and sarcopenia. Some studies show lower plasma concentrations of branched-chain amino acids (BCAAs) in elderly subjects (16,17), whereas others do not (18,19). This may be due to the increased first-pass splanchnic extraction of amino acids in older people, with a consequent decrease in delivery to the skeletal muscle tissue and availability for muscle tissue anabolism (20). Most kinetics studies show no difference in the ability of older subjects to retain and metabolise BCAAs (21–24).

These observations suggest that the dietary requirement of proteins and essential amino acids is higher in the elderly than in young adults (25) and that an increased intake of a mixture of amino acids or essential amino acids can increase amino acid availability and result in the stimulation of muscle protein anabolism (26).

A number of reports, including a recent well conducted meta-analysis concludes that dietary supplement of essential amino acids is more effective than non-essential amino acid or whole protein supplementations in malnourished patients (27).

Notably, amino acid mixtures enriched in BCAAs have been shown to promote mitochondrial biogenesis and function, in addition to decrease oxidative stress via nitric oxide (NO) and mechanistic target of rapamycin (mTOR) signalling pathways in middle-aged mice (28). A more recent study has shown the stimulatory effect of leucine on mitochondrial respiration and ATP production in human macrophages (29). These results are important because mitochondrial dysfunction is a hallmark of the aging processes and age-related disorders, including sarcopenia and cognitive decline, are characterized by reduced mitochondrial mass and function [for a comprehensive review, see N. Sun et al, 2016 (30)]. Dietary supplementation of BCAA-enriched mixtures (BCAAem) may contribute to slow-down mitochondrial decline and to ameliorate clinical status of malnourished elderly patients (31).

The MATeR study thus aimed to evaluate the efficacy of a specific BCAAem compared to diet advice to promote mitochondrial function and improve clinical outcomes, particularly muscle and cognitive performance, in malnourished elderly community-dwelling subjects.

Materials and methods

Study design.

We conducted a parallel, randomized, controlled, open-label trial to determine the efficacy of dietary BCAAem supplementation, as compared with diet advice, in the slow-down of both muscle and cognitive deficit in malnourished community-dwelling men and women aged 80 years or older. Randomisation was performed by computer generated tables to allocate treatments, with a simple randomization method; the patients received a consecutive number after enrolment and were subsequently allocated to randomization list, according with Kim et al (32). The randomisation was carried out by the principal investigator, scientists performing lab measurement and statistical analyses were blind to treatment.

The inclusion criterion was malnutrition defined as MNA lower than 17. The MNA test is composed of 18 items that can be completed in less than 10 minutes. It provides a multidimensional assessment of senior patients nutritional status taking into account four domains: anthropometry, general status, dietary habits, and self-perceived health and nutrition states. Anthropometry includes the measurement of calf and arm circumferences, Body Mass Index (BMI), calculated after the measurement of weight and height, and questions about weight loss (4 items); general status comprehends 7 questions related to general health, medication and mobility; the assessment of dietary habits comprehends 5 questions on the number of meals, food and fluid intake and autonomy of feeding; 2 questions evaluate self-perceived health and nutrition states (33,34).

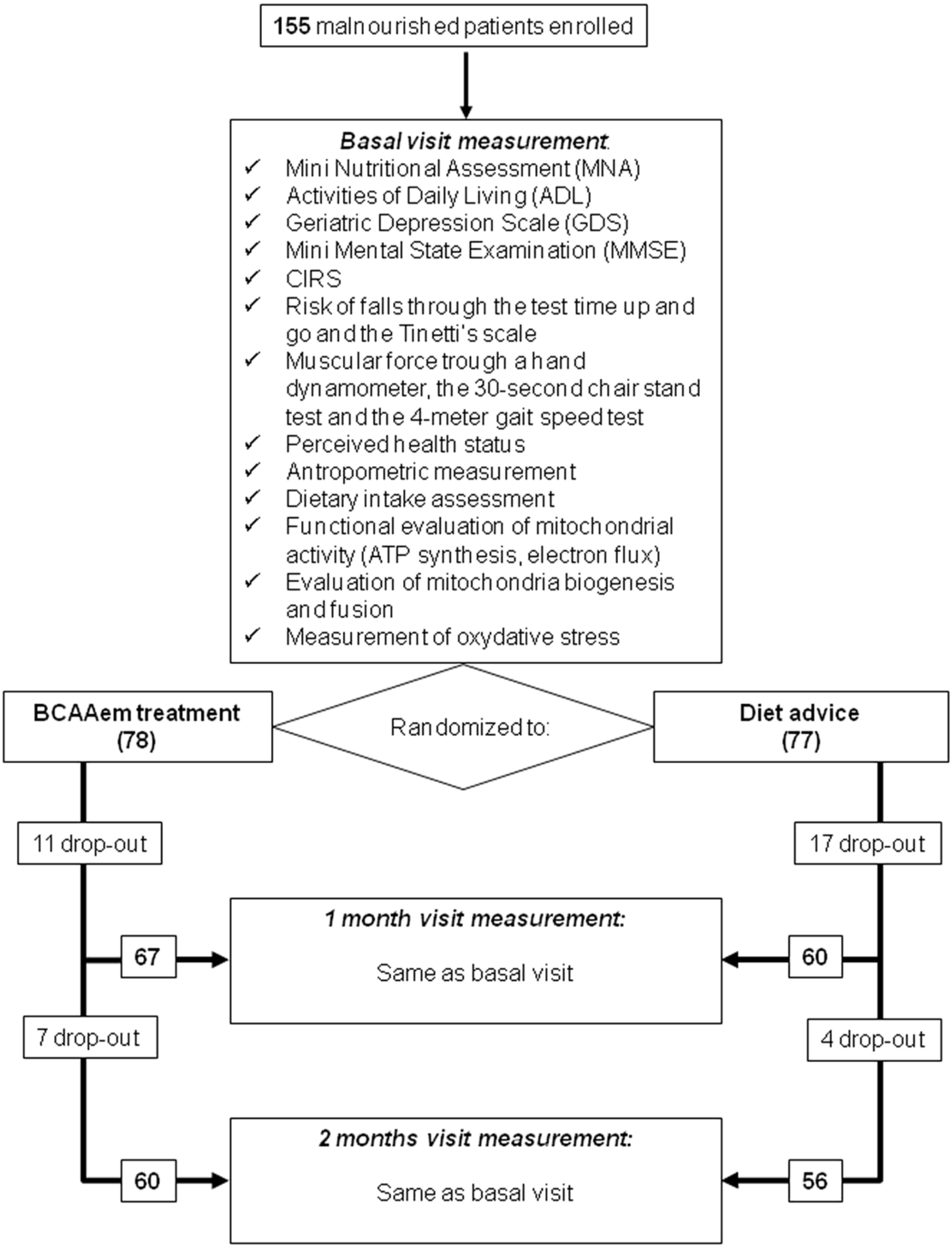
Exclusion criteria were known malignancy, life expectancy of less than two months, heart failure (NYHA IV), end stage renal disease, liver cirrhosis (Child B-C), tube/percutaneous endoscopic gastrostomy feeding or parenteral nutrition, Mini-Mental State Examination (MMSE)≤18 and MNA>17. MMSE ≥ 18 identifies patients with mild form of cognitive impairment, those patients generally do not have problems in swallowing and are able to take drugs. We evaluated for inclusion 336 malnourished patients presenting to our out-patients service for a geriatric evaluation, the evaluation was done by expert geriatricians, of the evaluated patients 181 were excluded for presence of exclusion criteria; one hundred and fifty-five malnourished elderly patients living at home in the community and who were admitted to the outpatients' department of our Unit were enrolled. Patients were evaluated at baseline and randomised to receive diet advice, summarised in an easy-to-use brochure for lay persons (77 patients) or to BCAAem supplements (78 patients, Aminotrofic®, kindly supplied by Errekappa Euroterapici S.p.A. and Professional Dietetics S.p.A, 2 sachets/day). Aminotrofic® is a BCAA enriched mixture, it contains Leucine (1,250 mg), Lysine (650 mg), Isoleucine (625 mg), Valine (625 mg), Threonine (350 mg), Cystine (150 mg), Histidine (150 mg), Phenylalanine (10 mg), Methionine (50 mg), Tyrosine (30 mg), Tryptophan (20 mg), Vitamin B 6 (0.1 mg), Vitamin B1 (0.15 mg). We suggested the patients to take the BCAAem in the mid-morning and afternoon, regardless of food ingestion. In order to check the compliance we asked the patients to bring back at center the empty sachets at follow-up visit.

Diet advice comprised general advice on the meaning and consequences of malnutrition and dietary recommendation based on the principle of "Food First" to maximize the patient nutritional intake from regular food and drink according with the ESPEN Guidelines on Enteral Nutrition for geriatric patients (35). According to the "Food First" approach we suggested the patients to increase the frequency of eating, maximize the nutrient and energy density of food and drink and fortify food with the addition of fats and sugars, suggested recommendations were given to the patients both orally by the physician and by the use of a brochure; provided dietary recommendations are summarized in the **Supplementary Table 1**.

Period of patient enrolment: February 2013-September 2017. Patients were called back to the centre and all the measurements were performed after 1 and 2 months. 116 patients completed the study (60 treated with BCAAem and 56 with diet advice). In the BCAAem group, 2 patients died after the first month, 1 was admitted to hospital within the first month, 10 patients did not return for the month-1 visit and 5 patients did not return for the month-2 visit for personal reasons. In the diet advice group, 1 patient died during the first month, 2 patients did not return for the month-1 visit since they were hospitalised, 14 patients did not return for the month-1 visit and 4 patients for the month-2 visit for personal reasons, there were no collateral effects. Only data from patients with complete follow up were included in the statistical analyses (**Fig. 1**).

Fig.1. Diagram of the study design.

The diagram shows the study design and the number of patients at each visit in bold. The tests performed at each visit are specified.



The main outcome measures were muscle mass, strength and performance and mitochondrial ATP production; secondary measurements were cognitive performance, nutritional status, health perceived status, mitochondrial biogenesis and activity in peripheral blood mononuclear cells (PBMCs). The measurements were carried out at baseline and after 1 and 2 months of treatment.

Clinical assessment.

Global clinical assessment: Self-sufficiency was measured by assessing the Katz Index of Activity of Daily Living (ADL), which evaluates overall performance in six functions: bathing, dressing, going to toilet, transferring, continence and feeding; cognitive performance was evaluated by MMSE that is a brief test used to routinely track cognitive changes in an individual both cognitively intact or with severe cognitive impairment over time. Patients' mood was evaluated by the short form of Geriatric Depression Scale (GDS), the scale consists of 15 questions related to the patient's mood answered "yes" or "no". The cut-off point adopted to define a patient as depressed is a GDS higher than 7 (36). Perceived health status was measured by asking the patients to answer the question "How is your health in general?". The patients' answers: "very good", "good", "fair", "bad" or "very bad" were rated from 5 (Very good) to 1 (Very bad), although there is not yet full standardisation of the measurement of perceived health status across Organisation for Economic Co-operation and Development (OECD) Countries, here we used a standard health interview survey instrument used in the OECD Health Statistics 2007 (37), confirmed in OECD Health Statistics 2018 (available on line at http://www.oecd.org/els/health-systems/health-data.htm) and accepted in Italy. The Cumulative Index Rating Scale (CIRS) was also recorded, this scale accounts for both the presence and the severity of co-morbidities (38).

Nutritional status assessment: We assessed patients' dietary intake using the PROGEO software (Progeo S.r.l., Italy), it provides an extensive food database and allows to record and to accurately estimate patients' average nutritional intake. The Photo Intake tool helps patients to recognize the amount of food by the visual weight method, ingested showing pictures of food in 3 portions, food quantities can also be recorded as conventional standard units (spoon, glass, cup, etc.). The software provides a large food database and automatically displays patients' average daily calories and nutrients intake. The interview was based on the recall method on 7 days making reference to the "standard week" as suggested by the manufacturer. The interview was done by geriatricians trained by a nutritionist.

BMI was assessed by weighing patients by a precision scale and measuring their height using an altimeter wall, BMI was calculated as weight in kg/height in meters squared. Percentage of fat mass was measured using a plicometer (Mahr GMBH Esslingen), the Pollock, Schmidt and Jackson's formula on three sites (triceps, subscapular and abdomen) was applied (39,40). Skinfold thickness measurements were performed by trained staff according to standard technique: the skinfold thickness was measured by lifting a fold of skin and subcutaneous fat away from the underlying muscle and bone, the skinfold thickness was measured in duplicate with the plicometer. When a difference between the first and the second measurement exceeded 6 mm, a third measurement was taken. The plicometer is applied 1cm from the ridge of skin; take reading 3 seconds after application, to standardise any effects produced by deformation of tissues. The triceps skinfold was measured at the back of the left arm, midway between the acromial process of the scapula and the olecranon process of the ulna. The subscapular skinfold is picked up just under the lower angle of the scapular lifted horizontally below the tip of right scapula. The abdominal skinfold was lifted diagonal midway between umbilicus and right anterior superior iliac spine.

Muscle mass, strength and performance: Appendicular muscle mass was measured using arm and calf circumference. Arm circumference was measured in duplicate to the nearest 0.001 m at a point midway between the lateral projection of the acromion process of the scapula and the inferior margin of the olecranon process of the ulna. The mean of the two measurements was used in the analyses. The calf circumference was measured to the nearest 0.001 m on the left leg with the participant standing straight, feet 20 cm apart, body weight equally distributed on both feet and at the level of the widest circumference of the calf; measurements were taken according to the Longitudinal Aging Study Amsterdam (LASA - http://www.lasa-vu.nl/themes/physical/anthropometry.htm).

Muscle strength was measured via the hand grip test, using a hydraulic hand dynamometer (MSD, Europe) (41) to assess muscle performance and mobility the Timed Up and Go (TUG) test, 30 seconds Chair Sit to Stand (30-s CST) test and the 4 meters gait speed test were performed.

TUG is a simple test used to assess a person's mobility and requires both static and dynamic balance. TUG is performed by measuring the time that the patient takes to rise from a chair, walk three meters, turn around, walk back to the chair and sit down. TUG performed in ten seconds or less indicate normal mobility; 11–20 seconds are within normal limits for frail, elderly and disabled patients and greater than 20 seconds means that the person needs assistance outside and indicates further examination and intervention. A score of 30 seconds or more suggests that the person may be prone to falls (42). The 30-s CST allows the evaluation of lower body strength and to assess the fatigue effect due to the number of sit-to-stand repetitions. It is performed with a chair without arms, the patient seated in the middle of the chair with the arms crossed over his/her chest, then is instructed to stand up as quickly as possible safely without using his/her arms. The number of stands the patients completed in 30 seconds is manually recorded (43).

The 4-meter gait speed test was performed using a stopwatch and measures the time, in seconds, the patients take to complete a 4-meter walk.

The risk of falls further was evaluated using the Tinetti Gait and Balance Instrument as follows: to test the patient's balance, the patient has to sit in a hard, armless chair and is asked to rise and stay standing, then turn 360° and sit back down. Next, the patient walks a few meters at a normal speed, turns, walks back and sits down. The evaluator observes several features and scores the patient's performance: the higher the score, the better the performance. The maximum score for Gait is 12 points, while the maximum for Balance is 16 points, with a total maximum for the overall Tinetti Instrument of 28 points. Score Interpretation: <19 high risk of falls, 19-28 low risk of falls.

Laboratory tests.

Functional evaluation of mitochondrial activity: In order to isolate mitochondrial fractions, blood cells were washed twice in ice-cold PBS, then lysed in 0.5 mL buffer A (50 mMTris, 100 mMKCl, 5 mM MgCl2, 1.8 mM ATP, 1 mM EDTA, pH 7.2), supplemented with protease inhibitor cocktail III (Calbiochem), 1 mM PMSF and 250 mMNaF. Samples were clarified by centrifuging at 650×g for 3 min at 4°C, and the supernatant was collected and centrifuged at 13000×g for 5 min at 4°C. This supernatant was discarded and the pellet containing mitochondria was washed in 0.5 mL buffer A and suspended in 0.25 mL buffer B (250 mM sucrose, 15 mM K2HPO4, 2 mM MgCl2, 0.5 mM EDTA, 5% w/v BSA). A 50 µL aliquot was sonicated and used for the measurement of protein content, as reported in Campia et al, 2009 (44); the remaining part was diluted to a protein concentration of 10 µg/µL and stored at −80°C until the use. The activity of Complex I–III was measured on 10 µL of non-sonicated mitochondrial samples (44), suspended in 0.59 mL buffer C (5 mM KH2PO4, 5 mM MgCl2, 5% w/v BSA). Then 0.38 mL buffer D (25% w/v saponin, 50 mM KH2PO4, 5 mM MgCl2, 5% w/v BSA, 0.12 mM cytochrome c-oxidized form, 0.2 mM NaN3) was added for 5 min at room temperature. The reaction was started with 0.15 mM NADH and was followed for 5 min. The absorbance was read using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, Winooski, VT). Results were expressed as nmol reduced cytochrome C/min/mg mitochondrial proteins. In each experimental set, the complex I inhibitor rotenone (100 µM) was added as an internal negative control. In the presence of rotenone, the electron flux was reduced to below 5%.

The amount of ATP was measured on 20 µg of mitochondrial extracts using the ATP Bioluminescent Assay Kit (FL-AA, Sigma Aldrich Co., St. Louis, MO). Data were converted into nmol/mg mitochondrial proteins, using a previously set calibration curve.

Under these experimental conditions, the rate of cytochrome C reduction, expressed as nmol cytochrome C reduced/min/mg cell protein, was dependent on the activity of both Complex I and Complex III.

Real time PCR and assessment of mitochondria biogenesis and fusion: Real time PCR (RT-PCR) was used to evaluate the mRNA levels of Cytochrome C Oxidase 1 and 4 (COX-1 and COX-4), Mitofusin-1 and 2 (MFN-1 and MFN-2), Nuclear Respiratory Factor-1 (NRF-1) and Mitochondrial Transcription Factor A (TFAM) from whole blood nucleated cells.

Red cells were lysed in all peripheral blood samples, total nucleated cells were collected and dissolved in TRIzol reagent (TRISure, Bioline Reagents Ltd, UK) and frozen at -80 °C until RNA extraction. RNA was isolated using chloroform extraction and subsequent isopropanol precipitation according to the manufacturer's protocol. 1 µg of RNA was reverse-transcribed to single-stranded cDNA using the SensiFAST cDNA Synthesis Kit (Bioline Reagents Ltd, UK). RT-PCR was performed using the SensiFAST SYBR Hi-ROX Kit (Bioline Reagents Ltd, UK). The housekeeping control gene was β-actin, and gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. The primers used (Invitrogen, California, USA) are shown in **Supplementary Table 2**.

All the lab experiments were performed in duplicate, data presented are averages of the duplicates. The coefficient of variation intra-operator ranges between 0.03 and 1.00 for all the measurements.

Oxidative stress: To determine the oxidative stress level, we measured plasma thiobarbituric acid reactive substances (TBARs), as indicators of lipid peroxidation, using ELISA (TBARS Assay Kit, Cayman Chemical, MI, USA), according to the manufacturer's protocol.

Statistical analyses: The sample size was calculated on both clinical and lab outcomes; amongst clinical outcome muscle mass was used; in particular sample size provide an 85% power (p<0.05), 50 patients per group have to be enrolled to detect a difference (alpha error = 0.05) of at least 2% variation in muscle mass, based on a study on the effect of BCAAs administration on muscle mass and performance in humans (45). As the patients were old and frail we assumed a possible 35% of drop out at the follow-up. In order to calculate sample size for lab tests we considered as significant an increase of 1.5 fold in ATP production as shown by D'Antona et al with BCAAem in aged mice (28), data on ATP production in humans by PBMCs derives from Avis et al (46) based on this analyses sample size was calculated to provide 95% power (p<0.05) to detect a 1.5-fold difference in ATP production was 13 patients per group .

All the analysed variables were tested for normality by the kurtosis test, TUG, 30-s CST, 4 meters walking test, TBARs, electron flux were non-Gaussian.

To evaluate possible differences between patients treated with BCAAem or diet advice at baseline the patients were compare by one-way ANOVA for Gaussian variables and by the Mann-Whitney U test for non-Gaussian ones. Gender was compared amongst patients treated with BCAAem or diet adviceby $\chi2$ test.

The effect of treatment was evaluated per protocol using the two-way ANOVA for repeated measurements for Gaussian variables, non-Gaussian variables were evaluated after logarithmic transformation. To evaluate possible influences of mitochondrial function on muscle and cognitive performance six linear regression models (GLM) were fitted, between ATP and electron flux and TUG, 30-s CST, Tinetti and 4 meters walking test, hand grip and MMSE, non-Gaussian variables were logarithmically transformed.

SPSS 24.0 were used for the analyses and p<0.05 was considered statistically significant. Graphs were drawn using GraphPad 7.0 for Windows.

Ethics Committee approval and consent to participate.

The study was approved by the Ethics Committee of our Hospital ("Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino - A.O. Ordine Mauriziano - A.S.L. TO1", protocol number 0002637), in accordance with the ethical standards of the Declaration of Helsinki and its subsequent amendments. Informed consent was obtained from all individual participants included in the study. The full protocol is available upon request to the corresponding author.

Results

Patients treated with BCAAem or diet advice were comparable for all the clinical variables analysed, this excludes possible selection bias that could influence our results (**Supplementary Table 3**). Compliance to BCAAem was good, none of the patients have a compliance lower than 75%, the compliance ranges between 75% and 90%.

Treatment significantly improves general health and cognitive performance.

Patients' general health measured by perceived health status equally improved in both treatment groups and significantly correlated with nutritional status (MNA: $R=0.50$, $p<0.0001$; fat percentage: $R=0.26$, $p=0.005$) at the end of the follow-up period. Also, the mood measured by GDS significantly improved. The level of independence was not significantly influenced by treatment.

Patients' overall cognitive performance measured by MMSE significantly improved in patients treated with BCAAem, not in patients treated with diet advice; MMSE significantly correlated with MNA ($R=0.28$, $p=0.002$) as well as GDS ($R=-0.32$, $p<0.0001$) at the end of the follow-up period (**Table 1**).

Treatment significantly improves nutritional status.

Patients adhered to the dietary recommendation as shown by the increased caloric intake. Caloric intake increased in both groups and was particularly consistent in the group treated with diet advice, where dietary recommendations were reinforced by the use of an easy-to-use illustrated brochure, in this group also protein intake was significantly higher. During treatment, we observed a significant improvement in nutritional status measured by MNA, fat mass and BMI in both groups (**Table 2**).

BCAAem treatment increases muscle mass, strength and performance.

Muscle mass measured by calf and arm circumferences significantly increased in both groups as well as muscular strength measured by hand grip strength (**Table 3**).

To evaluate whether muscular performance was influenced by treatment, we performed the TUG, the 30-s CST test to evaluate both performance and resistance to fatigue. Muscular performance improved with treatment as did muscle mass. Risk of falls was measured using the Tinetti scale and the TUG, mobility was measured using the 4-meter gait speed test, these tests improved equally in the two groups (**Table 3**).

BCAAem improve bioenergetic capacity of PBMCs.

Here we show that ATP production and electron flux significantly increased over time only in mitochondria from patients treated with BCAAem, and that BCAAem maintain oxidative stress at baseline values, whereas, in patients treated with diet advice, oxidative stress increased over time (**Table 4**).

To model the relationship between mitochondria stimulation on PBMCs and effects of BCAAem or diet advice on muscular and cognitive performance we applied a linear regression approach.

Our data showed that mitochondrial ATP production significantly predicts balance measured by Tinetti after 2 months of treatment and 4 meters walking test after 1 month of treatment (**Table 5**). MMSE is significantly predicted by ATP after 1 month and by electron flux after 2 months of treatment (**Table 6**).

We also evaluated the effect of BCAAem treatment on some of the main mitochondrial biogenesis and fusion markers. Our data showed that treatment increases the expression of COX-1 and COX-4 and TFAM, whereas NRF-1 shows only a non-significant trend towards the increase, significant differences versus baseline levels were measured only in patients treated with BCAAem. The expression of MFN-1 and MFN-2 was increased although not significantly by treatment; in the BCAAem treated patients we observed an increased expression of these two molecules after one month of therapy (**Table 7**).

Discussion

As life expectancy increases, adequate nutrition is fundamental for successful aging, here, we confirm that amelioration of nutritional status is associated with improvement in general health status, muscle and cognitive performance in old malnourished patients, and show that this may be due to an improvement of their mitochondrial bioenergetics profile and decreasing oxidative stress.

Here we show that the diagnosis of malnutrition and its treatment, albeit using different approaches, is fundamental in improving patients' general health and nutritional status. Indeed, in both our treatment groups, there was an improvement in MNA, and weight gain, however the increase in caloric and protein intake was higher in patients treated only with diet advice; the use of this approach instead of the use of an isonitrogenous mix of non-essential aminoacids with a double blind design may be considered as a limitation of the study, however the use of the "food first" approach underlines the role of the physician's counselling in patients' adherence to diet. Another possible limitation of the study is the use of a "non-standard" recall method for diet evaluation, here we use a recall on the 7 days before the interview as our old patients usually follow a standard diet with little variation day by day, the evaluation of 7 days allow us to make a more comprehensive evaluation asking the patients "what is your usual meal (breakfast, lunch, dinner and snacks)? Do you change your meal during one week? If yes when and how during the previous week?". The improvement in cognitive performance, measured by MMSE, was significant in patients taking BCAAem supplements: indeed, patients treated with BCAAem gain, on average, 1.2 points of MMSE. This result is in accordance with a previous study in a cohort of patients with severe chronic obstructive pulmonary disease. However, in this study, the increase in cognitive performance was associated with improved pulmonary gas exchange and there was no mechanistic explanation (47). The effect of the administration of BCAAs in cognitive function was also previously assessed in severely brain damaged patients with hepatic encephalopathy or traumatic brain injuries: in these patients, intravenous BCAAs improve consciousness, assessed using the Glasgow Coma Scale and performance, assessed using the Disability Rating Scale. However, no cognitive tests were performed in these studies and the underlying mechanism was not investigated (48–52). A mechanistic explanation has been provided by the study of animal models of brain injury, demonstrating the efficacy of dietary supplementation with BCAAs in promoting cognitive performance, by restoring hippocampal function, given that BCAAs act as glutamate and GABA precursors (53). In humans, a recent, very large retrospective study showed an association between serum level of isoleucine, leucine and valine with a lower risk of dementia. This study does not report any causal mechanism (54).

Poor nutrition and, in particular, protein-energy deficit further accelerate the loss of muscle mass and function associated with age: sarcopenia (55,56). Sarcopenia increases the risk of falls, disability, frailty, loss of independence and death increasing healthcare costs (57,58). Here, we show that intervention on malnutrition can improve muscle mass and performance. In particular, we show an improvement in balance by the Tinetti balance test and the TUG test, with a consequent reduction of the risk of falls and fall-related injuries in a population with increased risk of falls (average TUG higher than 14 seconds). Treatment increases muscle performance, increasing mobility, and resistance to fatigue; the improved performance in the 4-meter gait speed test observed during treatment demonstrates an overall increase in health and the performance status of the patients according to previous study (62). Also, muscle strength measured using hand dynamometer increased in both treatment groups.

Taken together, these data demonstrate that dietary intervention can counteract the decrease in physical and cognitive performance in old malnourished patients and that loss of muscle mass and function can be countered using an appropriate treatment strategy in a rapidly aging population.

Several studies suggest a major role of mitochondrial dysfunction as a major contributor to aging and age-related diseases [for a review, see Cedikova et al, 2016 (63)]. Mitochondrial biogenesis, ATP production and oxidative phosphorylation capacity decrease with aging and production of reactive oxygen species, damaged mitochondrial DNA and protein increase [for a review, see Cedikova et al, 2016 (63)]. In this study, we further investigate the mechanisms underlying the better clinical effects obtained with the administration of BCAAem, besides diet advice, by evaluating the treatment effects on mitochondrial function, biogenesis and fusion, as well as oxidative stress. In animal models, the administration of BCAAs has been shown to be effective in increasing the number and the function of mitochondria (28). In a small cohort of young healthy obese and non-obese subjects, the infusion of a mixture of aminoacids increased ATP production rates of muscular mitochondria in lean, but not in obese subjects (64). The increase in size, number and energy production of mitochondria that follows the administration of BCAAem was associated with better muscle performance, on both at skeletal and cardiac muscle level in mice (28). Here we evaluated mitochondria from PBMCs, it has been previously shown that mitochondria isolated from skeletal muscle and from PBMCs have a similar bioenergetics profile and are associated with gait speed in older adults (65), as regards neurological tissues other authors measured mitochondrial function in PBMCs and correlates its reduction with neurodegeneration (66). According with these observations we show that mitochondrial activity measured using ATP production and electron flux increase is associated with both cognitive and muscular performance amelioration in elderly patients; these data are particularly interesting as they suggest a possible non-invasive and reliable measure to follow up treatment outcomes. We show that the increase of TFAM, mitochondrial respiratory chain (COX-1 and COX-4) and mitofusin gene expressions correlate with an increase in ATP production and electron flux, and suggest that BCAAem treatment induces biogenesis, activity and fusion of mitochondria, stimulating at the end the bioenergetic capacity of PBMCs.

Mitochondrial biogenesis and fusion are particularly increased after the first month of treatment. Afterwards, there is a plateau with no further increase, whereas mitochondrial activity increases during the entire follow-up period. This suggests that, after an increase in the number and fusion of mitochondria, the organelles maintain an increased function without any further increase in their number.

Other than the effect on muscle mass and function, treatment of malnutrition is associated with improved general health. This may be due to better energy production associated with an increase in respiratory chain activity and a decrease in oxidative stress, in an experimental model of progeroid aging characterised by increased DNA damage, boosting mitochondrial preserves mammalian health and increases longevity (67).

It is well known that oxidative damage is one of the components of aging: the increase in free radical production and the decrease in the defence against oxidative stress cause molecular alteration and functional decay; the free radical theory of aging suggests that oxidative stress is an important factor in age-associated diseases (68). Here, we show that BCAAem supplementation lowers the levels of oxidative stress in elderly patients.

In conclusion, this study, for the first time, suggests that BCAAem treatment in old malnourished patients could be a good strategy able to ameliorate the bioenergetic capacity of PBMCs, this effect may partially explain the positive trend on muscle and cognitive performance in these patients.

Conflict of interest statement and funding

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Statement of authorship

FS and IB performed the lab experiments, acquired and analysed the lab data, and participated in drafting and critically revising the manuscript. CRavetta, GC, FD, CF, FGP and PP performed the clinical evaluation of patients and managed the dataset. MM, EN, CRiganti, CRuocco and GCI participated in the study design and were major contributors in writing the manuscript. PD designed the study, performed the statistical analyses and wrote the paper. All authors read and approved the final manuscript.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

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Table 1. Global clinical assessment at baseline and follow-up according to treatment.

The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as mean ± SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

* Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2 months; $ Denotes differences between 1 and 2 months; Significant values are in bold.

	Perceived health status					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	3±0.1	**-0.56 to -0.02***	2.9±0.1	**-0.56 to -0.02***	**Time**	**< 0.0001**
1 month	3.3±0.1	-0.5 to 0.02$	3.2±0.1	-0.6 to -0.04$	**Treatment**	0.8437
2 months	3.5±0.1	**-0.8 to -0.3****	3.5±0.2	**-0.8 to -0.3****	**Interaction**	0.9300
	MMSE[2]					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	25.1±0.4	**-1.1 to -0.04***	25.6±0.4	-0.2 to 0.8*	**Time**	**0.0139**
1 month	25.7±0.4	-0.8 to 0.2$	25.3±0.5	-1.0 to 0.06$	**Treatment**	0.9422
2 months	26±0.4	**-1.4 to -0.3****	25.8±0.4	-0.7 to 0.4**	**Interaction**	**0.0171**

	GDS[3]					
	BCAAem[1]		Diet advice		Two-way ANOVA	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	6.1±0.3	-0.4 to 0.8*	6.0±0.4	-0.2 to 0.9*	**Time**	**0.0084**
1 month	5.9±0.4	-0.3 to 0.9$	5.7±0.4	-0.3 to 0.8$	**Treatment**	0.7448
2 months	5.6±0.4	-0.1 to 1.1**	5.4±0.3	**0.01 to 1.2****	**Interaction**	0.9184

	ADL[4]					
	BCAAem[1]		Diet advice		Two-way ANOVA	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	10.2±0.3	-0.2 to 0.4*	9.7±0.3	-0.2 to 0.5*	**Time**	0.3569
1 month	10.1±0.3	-0.2 to 0.5$	9.5±0.3	-0.5 to 0.1$	**Treatment**	01130
2 months	10.0±0.3	-0.1 to 0.6**	9.7±0.3	-0.3 to 0.30**	**Interaction**	0.2294

[1]Branched Chain Amino Acid Enriched Mixture; [2]Mini-Mental State Examination; [3]Geriatric Depression Scale; [4]Activity of Daily Living.

Table 2. Patients' nutritional status at baseline and follow-up according to treatment.

The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as mean ± SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

* Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2 months; $ Denotes differences between 1 and 2 months; Significant values are in bold.

	Caloric Intake (Kcal/day)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1095±36	**-137 to -15.8***	1042±29	**-169 to -48***	**Time**	**<0.0001**
1 month	1172±36	-80 to 42.4$	1151±31	**-168 to -47$**	**Treatment**	0.9740
2 months	1189±36	**-156 to -34.1****	1259±38	**-277 to -156****	**Interaction**	**0.0031**

	Daily protein Intake (g/Kg weight)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	0.85±0.02	-0.03 to 0.09*	0.87±0.02	**-0.20 to -0.04***	**Time**	0.0874
1 month	0.83±0.02	-0.06 to 0.06$	0.97±0.02	-0.04 to 0.08$	**Treatment**	**0.0001**

2 months	0.83±0.02	-0.03 to 0.09**	0.96±0.02	**-0.14 to -0.02****	**Interaction**	**0.0007**
	MNA[2]					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	14.8±0.26	-0.03 to 0.09*	14.9±0.28	**-0.16 to -0.04***	**Time**	**<0.0001**
1 month	18.0±0.43	-0.06 to 0.06$	17.8±0.38	-0.04 to 0.08$	**Treatment**	0.9057
2 months	18.9±0.5	-0.03 to 0.09**	18.8±0.47	**-0.14 to -0.02****	**Interaction**	0.8366
	Fat mass (%)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	18.8±0.94	-1.68 to 0.61*	20.2±0.88	-1.7 to 0.6*	**Time**	**0.0009**
1 month	19.3±0.92	-2.16 to 0.13$	20.8±0.88	-1.6 to 0.7$	**Treatment**	0.1708
2 months	20.3±0.94	**-2.70 to -0.41****	21.2±0.88	-2.2 to 0.1**	**Interaction**	0.6438
	BMI[3]					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*

baseline	20.5±0.42	-1.42 to 0.10*	20.8±0.41	-1.38 to 0.14*	**Time**	**0.0010**
1 month	21.2±0.68	-0.92 to 0.60$	21.5±0.42	-0.81 to 0.71$	**Treatment**	0.7239
2 months	21.3±0.66	**-1.57 to -0.05****	21.5±0.41	-1.43 to 0.09**	**Interaction**	0.9482

[1]Branched Chain Amino Acid Enriched Mixture; [2]Mini Nutritional Assesment test; [3]Body Mass Index.

Table 3. Muscle mass, strength and performance at baseline and follow-up according to treatment.

The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as mean ± SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

* Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2 months; $ Denotes differences between 1 and 2 months; Significant values are in bold.

	Muscle mass					
	Calf circumference (cm)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	30.4±0.35	**-1.14 to -0.04***	30.7±0.43	-1.08 to 0.02*	**Time**	**0.0004**
1 month	31.0±0.38	-0.87 to 0.23$	31.2±0.40	-0.54 to 0.56$	**Treatment**	0.8560
2 months	31.3±0.39	**-1.45 to -0.36****	31.19±0.39	-1.07 to 0.03**	**Interaction**	0.4521
	Arm circumference (cm)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	22.7±0.36	-0.75 to 0.23*	23.0±0.40	-0.83 to 0.15*	**Time**	**0.0045**
1 month	23.0±0.37	-0.77 to 0.21$	23.3±0.40	-0.54 to 0.44$	**Treatment**	0.6754

2 months	23.3±0.38	**-1.03 to -0.04****	23.4±0.44	-0.88 to 0.10**	**Interaction**	0.7351

Muscle strength

	Hand grip (Kg)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	17.9±1.0	-2.01 to 0.47*	17.9±1.0	-1.84 to 0.65*	**Time**	**0.0474**
1 month	18.5±1.0	-1.6 to 0.87$	18.7±0.9	-1.0 to 1.50$	**Treatment**	0.7796
2 months	18.3±1.0	-2.40 to 0.10**	19.1±1.0	-1.59 to 0.90**	**Interaction**	0.5231

	TUG (sec)[2]					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	19.8±2.14	**1.5 to 7.6***	20.5±1.5	-1.2 to 4.9*	**Time**	**0.0001**
1 month	15.2±1.0	-2.9 to 3.2$	18.7±1.6	-2.1 to 4.0$	**Treatment**	0.2780
2 months	15.1±1.1	**1.6 to 7.8****	17.7±1.7	-0.3 to 5.9**	**Interaction**	0.3215

	30-s CST[3]					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of*	*Mean±SE*	*95% CI of*	*Effect of*	*p*

		difference		difference		
baseline	6.8±0.5	**-2.6 to -0.7***	6.0±0.5	**-2.4 to -0.5***	**Time**	**<0.0001**
1 month	8.4±0.6	-1.0 to 0.88$	7.4±0.5	-1.6 to 0.3$	**Treatment**	0.3328
2 months	8.5±0.7	**-2.7 to -0.7****	8.1±0.6	**-3.0 to -1.1****	**Interaction**	0.5810
	Tinetti					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	20.4±0.8	**-2.1 to -0.1***	18.3±0.8	**-3.2 to -1.1***	**Time**	**< 0.0001**
1 month	21.5±0.7	-1.7 to 0.3$	20.4±0.8	-1.3 to 0.7$	**Treatment**	0.1503
2 months	22.2±0.7	**-2.8 to -0.8****	20.7±0.9	**-3.4 to -1.4****	**Interaction**	0.2076
	4 meters walking test (sec)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	8.2±0.6	-0.3 to 1.7*	9.8±0.7	**0.4 to 2.3***	**Time**	**<0.0001**
1 month	7.5±0.6	-0.7 to 1.3$	8.4±0.7	-0.6 to 1.4$	**Treatment**	0.1684
2 months	7.2±0.6	**0.04 to 2.0****	8.0±0.7	**0.8 to 2.8****	**Interaction**	0.3955

681 [1]Branched Chain Amino Acid Enriched Mixture; [2]Timed Up and Go test; [3]30 seconds Chair Sit to Stand test.

Table 4. Mitochondrial activity and oxidative stress at baseline and follow-up according to treatment.

The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as mean ± SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

* Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2 months; $ Denotes differences between 1 and 2 months; Significant values are in bold.

	ATP (changes vs baseline)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.00±0.00	**-0.45 to -0.15***	1.00±0.00	-0.13 to 0.17*	**Time**	**0.0001**
1 month	1.30±0.09	-0.28 to 0.016$	0.98±0.01	-0.16 to 0.14$	**Treatment**	**0.0005**
2 months	1.43±0.10	**-0.58 to -0.28****	0.99±0.02	-0.14 to 0.16**	**Interaction**	**0.0001**
	Electron flux (changes vs baseline)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.00±0.00	**-0.38 to -0.13***	1.00±0.00	-0.13 to 0.13*	**Time**	**< 0.0001**
1 month	1.26±0.05	**-0.37 to -0.12$**	1.00±0.01	-0.14 to 0.12$	**Treatment**	**< 0.0001**

2 months	1.50±0.09	**-0.62 to -0.38****	1.01±0.04	-0.14 to 0.12**	**Interaction**	**< 0.0001**
	TBARs[2] (µg/M)					
	BCAAem[1]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	2.3±0.4	-2.8 to 1.2*	4.1±0.7	-3.02 to 0.97*	**Time**	**0.0007**
1 month	3.0±0.57	-2.4 to 1.6$	4.5±0.9	**-4.61 to -0.61$**	**Treatment**	**0.0289**
2 months	3.2±0.70	-3.1 to 0.85**	6.7±1.3	**-5.64 to -1.64****	**Interaction**	**0.0332**

689 [1]Branched Chain Amino Acid Enriched Mixture; [2]Thiobarbituric Acid Reactive Substances.

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Table 5. Muscle performance and balance.

The table shows the results for linear regression models (GLM), non-Gaussian variables indicated by * were logarithmically transformed. Significant values are in bold.

Dependent variable	Co-variate	β±SE	t	p	95% CI
Tinetti baseline	*Slope*	18.9±2.7	7.0	**0.000**	**13.5 to 24.4**
	Electronflux baseline	-0.02±0.01	-1.4	0.179	-0.05 to 0.009
	ATP baseline	0.05±0.03	1.5	0.134	-0.016 to 0.119
Tinetti 1 month	*Slope*	20.0±2.9	6.8	**0.000**	**14.1 to 25.8**
	*Electronflux 1 month**	0.000±0.013	0.02	0.984	-0.03 to 0.26
	ATP 1 month	0.011±0.04	0.32	0.752	-0.06 to 0.08
Tinetti 2 months	*Slope*	48.9±13.5	3.6	**0.001**	**21.8 to 75.9**
	*Electronflux 2 months**	0.05±0.04	1.5	0.131	-0.02 to 0.122
	ATP 2 months	-13.5±6.2	-2.2	**0.033**	**-25.9 to -1.1**
Dependent variable	**Co-variate**	**β±SE**	**t**	**p**	**95% CI**
4 meters walking test baseline*	*Slope*	0.89±0.08	11.2	**0.000**	**0.7 to 1.0**
	Electronflux baseline	0.000±0.000	0.8	0.394	0.000 to 0.001
	ATP baseline	-0.001±0.001	-1.4	0.175	-0.003 to 0.001

4 meters walking test 1 month*	*Slope*	0.96±0.10	9.6	**0.000**	**0.76 to 1.16**
	*Electronflux 1 month**	0.000±0.000	0.40	0.691	-0.001 to 0.001
	ATP 1 month	-0.002±0.001	-2.0	**0.045**	**-0.005 to $-6.1E^{-5}$**
4 meters walking test 2 months*	*Slope*	0.42±0.46	0.92	0.361	-0.49 to 1.33
	*Electronflux 2 months**	-0.002±0.001	-1.73	0.089	-0.004 to 0.000
	ATP 2 months	0.23±0.21	1.08	0.283	-0.19 to 0.644

Table 6. Cognitive performance.

The table shows the results for linear regression models (GLM), non-Gaussian variables indicated by * were logarithmically transformed. Significant values are in bold.

Dependent variable	Co-variate	β±SE	t	p	95% CI
MMSE[1] baseline	*Slope*	24.3±1.4	17.2	**0.000**	**21.5 to 27.2**
	Electronflux baseline	0.002±0.007	0.22	0.829	-0.01 to 0.02
	ATP baseline	0.02±0.018	0.99	0.327	-0.02 to 0.05
MMSE[1] 1 month	*Slope*	22.7±0.17	12.9	**0.000**	**19.2 to 26.20**
	*Electronflux 1 month**	-0.002±0.08	-0.27	0.790	-0.02 to 0.013
	ATP 1 month	0.048±0.02	2.29	**0.026**	**0.006 to 0.09**
MMSE[1] 2 months	*Slope*	29.9±7.9	3.8	**0.000**	**14.01 to 45.7**
	*Electronflux 2 months**	0.05±0.02	2.5	**0.014**	**0.01 to 0.09**

	ATP 2 months	-3.31±3.6	-0.9	0.366	-10.6 to 3.9

[1]Mini-Mental State Examination.

Table 7. Mitochondria biogenesis and fusion at baseline and follow-up according to treatment. The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as mean ± SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

* Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2 months; $ Denotes differences between 1 and 2 months; Significant values are in bold.

	COX-1[1] (changes vs baseline)					
	BCAAem[2]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.0±0.0	**-23.8 to -1.9***	1.0±0.0	-11.4 to 10.6*	**Time**	0.1155
1 month	13.9±8.5	-4.4 to 17.5$	1.4±0.3	-13.2 to 8.7$	**Treatment**	0.1967
2 months	7.3±3.6	-17.3 to 4.7**	3.7±1.2	-13.6 to 8.3**	**Interaction**	0.1409
	COX-4[3] (changes vs baseline)					
	BCAAem[2]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.0±0.0	**-2.3 to -0.10***	1.0±0.0	-1.39 to 0.76*	**Time**	**0.0459**
1 month	2.2±0.7	-0.7 to 1.47$	1.3±0.09	-1.1 to 1.04$	**Treatment**	0.2373

2 months	1.8±0.5	-1.9 to 0.3**	1.3±0.19	-1.4 to 0.73**	**Interaction**	0.3786
	TFAM[4] (changes vs baseline)					
	BCAAem[2]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.0±0.0	**-6.9 to -0.6***	1.0±0.0	-3.8 to 2.5*	**Time**	**0.0178**
1 month	4.8±1.0	-2.4 to 3.9$	1.7±0.5	-4.5 to 1.8$	**Treatment**	0.0932
2 months	4.2±1.15	-6.2 to 0.1**	3.0±1.5	-5.1 to 1.2**	**Interaction**	0.2235
	NRF-1[5] (changes vs baseline)					
	BCAAem[2]		**Diet advice**		**Two-way ANOVA**	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.0±0.0	-20.2 to 3.5*	1.0±0.0	-13.5 to 10.2*	**Time**	0.6599
1 month	9.4±6.4	-14.1 to 9.6$	2.6±0.7	-12.8 to 10.9$	**Treatment**	0.3507
2 months	11.6±9.5	-22.4 to 1.3**	3.6±1.2	-14.4 to 9.3**	**Interaction**	0.2055

	MFN-1[6] (changes vs baseline)					
	BCAAem[2]		Diet advice		Two-way ANOVA	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.0±0.0	**-22.4 to -2.1***	1.0±0.0	-10.8 to 9.4*	**Time**	0.0746
1 month	13.2±7.7	-7.0 to 13.3$	1.7±0.3	-10.3 to 10.0$	**Treatment**	0.1648
2 months	10.1±6.1	-19.3 to 1.0**	1.8±0.3	-11.0 to 9.3**	**Interaction**	0.1320

	MFN-2[7] (changes vs baseline)					
	BCAAem[2]		Diet advice		Two-way ANOVA	
	Mean±SE	*95% CI of difference*	*Mean±SE*	*95% CI of difference*	*Effect of*	*p*
baseline	1.0±0.0	**-11.6 to -1.1***	1.0±0.0	-6.0 to 4.5*	**Time**	0.0772
1 month	7.3±4.1	-1.9 to 8.6$	1.7±0.3	-5.9 to 4.6$	**Treatment**	0.2046
2 months	3.9±1.6	-8.2 to 2.3**	2.4±0.5	-6.6 to 3.9**	**Interaction**	0.1810

[1]Cytochrome C Oxidase-1; [2]Branched Chain Amino Acid Enriched Mixture; [3]Cytochrome C Oxidase- 4; [4]Mitochondrial Transcription Factor A; [5]Nuclear Respiratory Factor-1; [6]Mitofusin-1; [7]Mitofusin-2.